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Previous Patent	Next Patent	Referring Patents			
Abstract	Bkgnd/Summ	Clms	Draw. Desc	Front	Full
				KWIC	Legal
				Refs	Cit
				Cls	

US PAT NO: 4,931,390 [IMAGE AVAILABLE]

L2: 1 of 1

CLAIMS:

CLMS(1)

I claim:

1. Recombinant plasmids containing a DNA sequence which codes for lysostaphin and which in transformant microbial hosts will express a gene encoding lysostaphin from *S. simulans* (NRRLB-2628).

CLMS(2)

2. Recombinant plasmids according to claim 1, in which the recombinant plasmid is selected from the group consisting of pRG5, pJP1, pDF8, and pRP1.

CLMS(3)

3. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pRG5.

CLMS(4)

4. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pJP1.

CLMS(5)

5. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pDF8.

CLMS(6)

6. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pRP1.

CLMS(7)

7. Transformant microorganism which produces lysostaphin, the microorganism being transformed by a recombinant plasmid containing a DNA sequence which codes for lysostaphin from *S. simulans* (NRRL B-2628).

CLMS(8)

8. Transformant microorganisms according to claim 7, in which the microorganisms are selected from the group consisting of *E. coli*, Yeast, *Streptomyces* spp., and *Bacillus* spp.

CLMS(9)

CLMS (10)

CLMS (11)

CLMS (12)

CLMS (13)

CLMS (14)

CLMS (15)

CLMS (16)

CLMS (17)

CLMS (18)

CLMS (19)

sequence

1: ccggaactcttgaatgttttagttttgaaaattccaaaaaaaaacctaactttcttaatat
61: gattcatattattttaacacaatcagttagaatttcaaaaatcttaaagtcaatttttga
121: gtgtgtttgtatatttcatcaaatcaatcaatattattttactttcttcacgttaaaa
181: aatgtaatatttataaaaaatatgctattctcataaagtcaataataaattaggaggatt
241: aaggttgagaaaaacaaaaacaattattatacgaagaccttttagctattggactgagta
f-MetLysLysThrLysAsnAsnTyrTyrArgProLeuAlaIleGlyLeuSerThr
301: atttgcccttagcatctattgtttatggagggattcaaatgaaacacatgcttctgaaa

PheAlaLeuAlaSerIleValTyrGlyGlyIleGlnAsnGluThrHisAlaSerGluLys
 361: aagtaatatggatgtttcaaaaaagtagctgaagtagagacttcaaaagccccagtaga
 SerAsnMetAspValSerLysLysValAlaGluValGluThrSerLysAlaProValGlu
 421: aaatacagctgaagtagagacttcaaaagctccagtagaaaaatacagctgaagtagagac
 AsnThrAlaGluValGluThrSerLysAlaProValGluAsnThrAlaGluValGluThr
 481: ttcaaaagctccagtagaaaaatacagctgaagtagagacttcaaaagctccagtagaaaa
 SerLysAlaProValGluAsnThrAlaGluValGluThrSerLysAlaProValGluAsn
 541: tacagctgaagtagagacttcaaaagctccggtagaaaaatacagctgaagtagagacttc
 ThrAlaGluValGluThrSerLysAlaProValGluAsnThrAlaGluValGluThrSer
 601: aaaagccccagtagaaaaatacagctgaagtagagacttcaaaagccctggttcaaaatag
 LysAlaProValGluAsnThrAlaGluValGluThrSerLysAlaLeuValGlnAsnArg
 661: aacagctttaagagctgcaacacatgaacattcagcacaatggttgaataattacaaaaa
 ThrAlaLeuArgAlaAlaThrHisGluHisSerAlaGlnTrpLeuAsnAsnTyrLysLys
 721: aggatattggttacggctccttatccattaggtataaatggcggtatgcactacggagttga
 GlyTyrGlyTyrGlyProTyrProLeuGlyIleAsnGlyGlyMetHisTyrGlyValAsp
 781: tttttttatgaattattggaacaccagtaaaagctatttcaagcggaaaaatagttgaagc
 PhePheMetAsnIleGlyThrProValLysAlaIleSerSerGlyLysIleValGluAla
 841: tggttgaggtaattacggaggaggtaatcaaataggtcttattgaaaatgatggagtgc
 GlyTrpSerAsnTyrGlyGlyGlyAsnGlnIleGlyLeuIleGluAsnAspGlyValHis
 901: tagacaatggtatatgcatctaagtaaatataatgttaaagtaggagattatgtcaaagc
 ArgGlnTrpTyrMetHisLeuSerLysTyrAsnValLysValGlyAspTyrValLysAla
 961: tgggtcaataatcggttggtctggaagcactggttattctacagcaccacatttacactt
 GlyGlnIleIleGlyTrpSerGlySerThrGlyTyrSerThrAlaProHisLeuHisPhe
 1021: ccaaagaatggttaattcatttttcaaattcaactgcccaagatccaatgcctttcttaa
 GlnArgMetValAsnSerPheSerAsnSerThrAlaGlnAspProMetProPheLeuLys
 1081: gagcgcaggatattggaagcaggtggtacagtaactccaacgccgaatacaggttgga
 SerAlaGlyTyrGlyLysAlaGlyGlyThrValThrProThrProAsnThrGlyTrpLys
 1141: aacaaacaaatatggcacactatataaatcagagtcagctagcttcacacctaatacaga
 ThrAsnLysTyrGlyThrLeuTyrLysSerGluSerAlaSerPheThrProAsnThrAsp
 1201: tataataacaagaacgactggtccatttagaagcatgccgcagtcaggagtctttaaagc
 IleIleThrArgThrThrGlyProPheArgSerMetProGlnSerSlyValLeuLysAla
 1261: aggtcaaacaattcattatgatgaagtgatgaaacaagacggtcatggttgggtaggtta
 GlyGlnThrIleHisTyrAspGluValMetLysGlnAspGlyHisValTrpValGlyTyr
 1321: tacaggtaacagtgccaacgtatttacttgctgtaagaacatggaataaatctactaa
 ThrGlyAsnSerGlyGlnArgIleTyrLeuProValArgThrTrpAsnLysSerThrAsn
 1381: tacttttaggtgttctttggggaactataaagtgagcgcgctttttataaacttatatgat
 ThrLeuGlyValLeuTrpGlyThrIleLys
 1441: aattagagcaataaaaaatttttctcattcctaaagttgaagc,
 (b) A DNA fragment coding for mature lysostaphin comprising nucleotides
 674-1411 in (a),
 (c) a DNA fragment coding for prepolysostaphin comprising nucleotides
 245-1411 in (a),
 (d) a DNA fragment coding for polysostaphin comprising nucleotides
 356-1411 in (a), and
 (e) a DNA fragment coding for the lysostaphin signal peptide comprising
 nucleotides 245-355 in (a).

CLMS (20)

20. The DNA fragment of claim 19 coding for mature lysostaphin.

CLMS (21)

21. The DNA fragment of claim 19 coding for prepolysostaphin.

CLMS (22)


22. The DNA fragment of claim 19 coding for polysostaphin.

CLMS (23)

23. The DNA fragment of claim 19 coding for the lysostaphin signal peptide.

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Previous Patent	Next Patent	Referring Patents			
Abstract	Bknd/Summ	Clms	Draw. Desc	Front	Full
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Abstract	Bkgnd/Summ	Clms	Draw. Desc	Front	Full
				KWIC	Legal
				Refs	Clt
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US PAT NO: 4,931,390 [IMAGE AVAILABLE] L2: 1 of 1
 DATE ISSUED: Jun. 5, 1990
 TITLE: Expression of the cloned lysostaphin gene
 INVENTOR: Paul A. Recsei, New York, NY
 ASSIGNEE: Public Health Research Institute of the City of New York, Inc.,
 New York, NY (U.S. corp.)
 APPL-NO: 07/034,464
 DATE FILED: Apr. 10, 1987
 REL-US-DATA: Continuation-in-part of Ser. No. 852,407, Apr. 16, 1986,
 abandoned.
 INT-CL: [5] C12N 9/00; C12N 21/02; C12N 21/00; C12N 15/00; C12N
 1/00; C12N 1/20; C12N 1/16; C12N 1/18; C07H 21/04
 US-CL-ISSUED: 435/183, 69.1, 220, 172.3, 243, 252.3, 252.31, 252.33,
 252.35, 255, 256, 320; 536/27; 935/11, 29, 69, 72, 73,
 74, 75
 US-CL-CURRENT: 435/183, 69.1, 220, 243, 252.3, 252.31, 252.33, 252.35, 254.2, 320.1,
 23.7
 SEARCH-FLD: 435/68, 70, 71, 91, 172.1, 172.3, 320, 183, 252.3, 252.31,
 252.35, 243, 255, 256, 220; 536/27; 935/9, 11, 14, 29,
 72-75
 REF-CITED:

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<u>4,467,036</u>	8/1984	Schnepf et al.	435/68
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 American Society for Microbiology, 1987.
 ART-UNIT: 185
 PRIM-EXMR: James Martinell
 LEGAL-REP: White & Case

ABSTRACT:

The present invention provides recombinant plasmids which is
 transformant microbial hosts express lysostaphin, a bacteriocin that
 kills most known staphylococcal species. The invention also provides
 lysostaphin, substantially free from non-lysostaphin contaminants.

Recombinant plasmids, pRG5, pJP1, pDF8 and pRP1, were derived by inserting a 1.5 kilobase segment of DNA coding for lysostaphin into the cloning vectors, pUC8, pBC16, pBD64 and pSPV1, respectively. E. coli strain JM105 transformed by pRG5 and members of Bacillus species, including B. subtilis and B. sphaericus transformed by pJP1, pDF8 and pRP1 produce lysostaphin which is immunologically and electrophoretically indistinguishable from that produced by S. simulans, the natural source. Furthermore, B. sphaericus strain 00/pJP1 transformants produce five times the amount of lysostaphin as S. simulans. The invention also provides the 1.5 kbp DNA fragment coding for lysostaphin. The sequence of the DNA encodes preprolysostaphin, a monomeric 389 amino acid protein, which is posttranslationally processed to mature lysostaphin.

23 Claims, 1 Drawing Figures

Main Menu	Search Form	Result Set	Show S Numbers	Edit S Numbers	First Hit
Previous Patent	Next Patent	Referring Patents			
Abstract	Bkgnd/Summ	Cims	Draw. Desc	Front	Full
				KWIC	Legal
				Refs	Cit
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23 Claims, 1 Drawing Figures

NO-PP-DRAWING: 1

BACKGROUND OF THE INVENTION

Lysostaphin is a bacteriocin secreted by a single known strain of *Staphylococcus simulans* originally isolated and named *Staphylococcus staphylolyticus* by Schindler and Schuhardt. The production of lysostaphin by *S. staphylolyticus* has been described previously in U.S. Pat. No. 3,278,378 issued Oct. 11, 1966 and in Proceedings of the National Academy of Sciences, Vol. 51, pp. 414-421 (1964). The single organism *S. staphylolyticus* (NRRL B-2628) which produces lysostaphin was recently identified as a biovar of *S. simulans* by Sloan et al., Int. J. System. Bacteriol., Vol. 32, pp. 170-174 (1982). Since the name *S. staphylolyticus* is not on the Approved List of Bacterial Names, the organism producing lysostaphin has been redesignated as *S. simulans*.

Lysostaphin production occurs during the stationary phase of *S. simulans* cultures grown under certain conditions and appears to be coordinated with production of other extracellular enzymes. Cultures that produce lysostaphin appear to be resistant to its activities while cultures grown under non-producing conditions are sensitive.

6/2/99 3:13 PM

with lysostaphin contaminated by non-lysostaphin immunogenic material from staphylococci might result in an undesirable, and potentially adverse, immunological response.

Lysostaphin isolated from culture filtrates of *S. simulans* has been characterized as a zinc-containing protein composed of a single polypeptide chain with a molecular weight of about 25,000 daltons. It is heat labile, nondialyzable and has an isoelectric point of about pH 11. Furthermore, the capacity of lysostaphin to lyse viable and heat-killed staphylococci and staphylococcal cell walls is destroyed by treatment with the enzyme trypsin.

Recombinant DNA techniques, whereby genes for a variety of proteins can be cloned by insertion into a plasmid, cosmid, or phage vector which can then be used to transform a microorganism, have been used widely to study the structure and expressions of genes and to produce a ready source of various pure proteins for various purposes. There have been, however, no reports relating to such cloning techniques being used to insert the gene encoding lysostaphin into a cloning vector to construct novel vectors which can transform a microorganism, other than *S. simulans* (NRRL B-2628), to allow the production of large amounts of lysostaphin.

BRIEF SUMMARY OF THE INVENTION

In accordance with the present invention, recombinant plasmids are described which in transformant microbial hosts will express a gene encoding lysostaphin. The recombinant plasmids were derived by inserting an identified DNA sequence which codes for lysostaphin into suitable cloning vectors.

Suitable cloning vectore include those which replicate in bacteria, including, inter alia, E. coli, Bacillus spp., and Streptomyces, and yeast. Host microorganisms include E. coli, Bacillus spp., Streptomyces, and yeast. The invention, however, is not limited to the above vectors and microbial hosts. It will be apparent to those skilled in the art that other vectors and hosts can be used in the practice of the invention.

In one embodiment, the DNA sequence coding for lysostaphin was inserted into the E. coli plasmid pUC8, a well-known cloning vector, to create recombinant plasmid pRG5. E. coli JM105 transformed by pRG5 produces lysostaphin.

In another embodiment of this invention, the lysostaphin gene from pRG5 was cloned into Bacillus plasmids pBC16, pBD64 and pSPV1 whereby recombinant plasmids, pJPI, pDF8 and pRP1, respectively were produced. Members of Bacillus species, including *B. subtilis*, transformed by one or another of these three recombinant Bacillus plasmids containing the gene for lysostaphin, secreted large amounts of lysostaphin into the culture medium. The invention further provides for *B. sphaericus* strain 00 which, when transformed by recombinant plasmid pJPI, produces about five times the amount of lysostaphin as can be obtained from cultures of *S. simulans*, (NRRL B-2628), the natural producer.

The lysostaphin expressed as a result of transformation of microbial hosts by the above-mentioned plasmids and other plasmids containing the lysostaphin gene is substantially free of non-lysostaphin contaminants, especially immunogenic staphylococcal contaminants.

The invention further provides a 1.5-kilobase pair (kbp) DNA fragment encoding lysostaphin, the sequence of such gene, and the 389 amino acid protein, preprolysostaphin, having a molecular weight of about 42,200 daltons which is encoded by the DNA fragment. The amino terminal sequence of preprolysostaphin contains a cluster of four positively

charged amino acid residues followed by an uncharged largely hydrophobic sequence and, therefore, has the properties of a signal peptide. Adjacent to the lysostaphin signal peptide is the prolysostaphin amino acid sequence. The "pro" sequence contains seven tandem repeats of a homologous 13 amino acid sequence which are removed during processing to mature enzyme.

The 1.5 kbp DNA fragment which contains the lysostaphin structural gene, therefore, codes for a preproenzyme protein (preprolysostaphin), which is subsequently processed to mature active lysostaphin having a molecular weight of approximately 26,920 daltons. It was heretofore unknown that lysostaphin was synthesized in precursor form which is subsequently processed to an active enzyme.

Also encompassed within the scope of this invention are DNA fragments which are homologous to the 1.5 kbp DNA fragment which codes for lysostaphin provided in Formula I and which code for functionally equivalent proteins.

The invention also provides for preprolysostaphin, prolysostaphin and lysostaphin, which is substantially free of non-lysostaphin immunogenic staphylococcal contaminants. The invention further encompasses those portions of the 1.5 kbp DNA fragment which code for the lysostaphin signal peptide, the prolysostaphin sequences, and the mature active lysostaphin, respectively. DNA fragments which are homologous to these three portions of the 1.5 kbp DNA fragment encoding lysostaphin and which encode functionally equivalent peptides are also within the scope of the invention.

DRAWING DESC:

BRIEF DESCRIPTION OF THE DRAWING

The present invention is now described with reference to the following detailed description, Examples and figures in which:

FIG. 1 is an immunoblotted electropherogram showing the production of lysostaphin and prolysostaphin by *E. coli*/pRG5 transformants and *S. simulans*.

DETDESC:

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides for recombinant plasmids which have been created by inserting the 1.5 DNA fragment which codes for lysostaphin into cloning vectors that replicate in various host microorganisms. Particularly preferred host organisms are *E. coli* and strains of *Bacillus subtilis*, *Bacillus sphaericus* and other *Bacillus* species. The 1.5 kbp DNA fragment is stably maintained and lysostaphin is expressed at high levels in cloned transformant bacteria harboring the plasmids. The 1.5 kbp DNA fragment coding for lysostaphin was isolated from *S. simulans* (NRRL-B-2628) and is present in this organism on a large penicillinase plasmid. A proenzyme with a molecular weight of about 42,200 daltons has now been shown to be produced by *S. simulans*. When the 1.5 kbp DNA fragment coding for lysostaphin is inserted into the plasmids of the present invention, lysostaphin expressed by the transformant microorganisms is secreted from the cells. Mature lysostaphin accumulates in large quantities in the medium in which the transformants are growing. Some of the genetically engineered transformant *Bacillus* strains produce considerably more lysostaphin per ml of culture supernatant than does *S. simulans*, the natural source of the enzyme.

The present invention, in particular, provides for plasmid, pRG5, which

was derived from the *E. coli* cloning vector pUC8 by inserting the 1.5 kbp DNA fragment coding for lysostaphin into the lacZ' gene of plasmid pUC8. Latel logarithmic phase cultures of *E. coli* strain JM105, which is the host bacteria for recombinant plasmids derived from pUC8, transformed by pRG5 had detectable levels of lysostaphin activity in the supernatant, periplasmic, and cytoplasmic fractions.

Furthermore, the present invention also particularly provides for recombinant plasmids into which the 1.5 kbp DNA fragment coding for lysostaphin has been inserted that can be used to transform *Bacillus* species. The invention thus provides for recombinant *Bacillus* plasmids pJP1, pDF8, and pRP1 which in a transformant host express lysostaphin. The plasmids were constructed by inserting the 1.5 kbp DNA fragment which codes for lysostaphin that is obtained from pRG5 into *Bacillus* plasmids pBC16, pBD64 and pSPV1 respectively. A further embodiment of this invention includes the transformant *Bacillus* species that produce and secrete lysostaphin upon transformation with these plasmids. Plasmids pJP1, pDF8, and pRP1 have been used to transform *B. subtilis* and *B. sphaericus*. The particularly preferred recombinant plasmid of this invention for use in *Bacillus* species is pJP1. The particularly preferred transformant host organisms expressing lysostaphin are competent *Bacillus subtilis* BD170 and *Bacillus sphaericus* 00 strains.

In particular, *B. sphaericus* strain 00 transformed by pJP1 produced at least five times the amount of lysostaphin per liter of culture as *S. simulans*, (NRRL B-2628). The recombinant product isolated from *B. sphaericus* strain 00/pJP1 is indistinguishable from the *S. simulans* lysostaphin on the basis of electrophoretic mobility, immunologic cross-reactivity with lysostaphin-specific antibodies, catalytic activity. Lysostaphin produced by transformant microorganisms according to this invention is substantially free of non-lysostaphin contaminants, in particular immunogenic staphylococcal contaminants.

The invention also provides for the cloned 1.5 kbp DNA fragment coding for lysostaphin, the sequence of which is provided in Formula I. The DNA sequence is characterized by an open reading frame extending from a TTG initiation codon at nucleotides 245-247 to a TGA termination codon at nucleotides 1412-1414 which encodes the 389 amino acid preprolysostaphin. It will be understood that DNA fragments homologous in sequence to the sequence in Formula I and which code for functionally equivalent proteins are within the scope of the invention.

E. coli JM105 carrying pRG5, ATCC Accession No. 67076; B. subtilis BD170 carrying pJP1, ATCC Accession No. 67078; B. sphaericus 00 carrying pJP1, ATCC Accession No. 67080; B. subtilis BD 170 carrying pDF8, ATCC Accession No. 67077; and B. subtilis BD 170 carrying pRP1, ATCC Accession No. 67079 are on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. USA.

The following examples are provided to illustrate the invention and are not intended to limit the same.

EXAMPLE 1

Construction of pRG5

Plasmid pRG5 was constructed by inserting the 1.5 kbp DNA fragment which codes for lysostaphin into the lacZ' gene of plasmid pUC8, an engineered cloning vector described by Vieira and Messing, Gene, Vol. 19, pp. 259-268 (1982).

Total DNA was isolated from *S. simulans* (NRRL B-2628) as follows:

S. simulans was grown to midlogarithmic phase in Casamino Acids medium described by Robinson et al. J. Bacteriol. Vol. 137, pp. 1158-1164

(1979). The cells were harvested by centrifugation, washed in Tris buffer (50 mM Tris, 50 mM EDTA, pH 7.8), and resuspended to 20% of the original culture volume in Tris buffer containing 50 μ g/ml lysostaphin (obtained from Mead-Johnson) and lysozyme (0.5 mg/ml). After a 2 hour incubation at 37.degree. C., pronase (1 mg/ml) and sodium dodecylsulfate (0.6%) were added and the suspension incubated a further 2 hours at 37.degree. C. The *S. simulans* lysate thus obtained by this treatment was extracted two times with an equal volume of phenol using standard techniques.

Nucleic acid was precipitated from the aqueous phase of the phenol-extracted lysate by the addition of two volumes of ice-cold 95% ethanol, collected by centrifugation, and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The dissolved nucleic acid was digested for 2 hours at 37.degree. C. with a combination of pancreatic ribonuclease (30 μ g/ml) and T1 ribonuclease (2 U/ml) to degrade any RNA present in the sample. The resulting DNA was again precipitated with ethanol and dissolved in TE buffer. Approximately 1.5 mg of *S. simulans* DNA was isolated from a 0.5 L culture of mid log phase cells.

Cloning was carried out using pUC8 as the vector and *E. coli* K12 strain JM105 as the host. Total *S. simulans* DNA, isolated as described above, was partially digested with Mbo I and fractionated by centrifugation through a 12 ml 10-30% sucrose gradient at 35,000 rpm for 20 hours. Ten μ g of DNA fragments ranging in size from 5-15 kilobase pairs (kbp), with a 10 kbp average size, were pooled and ligated to two μ g of Bam HI-digested pUC8. This plasmid confers ampicillin resistance and carries the lacZ' gene which codes for the amino terminal portion of *E. coli* beta-galactosidase. Insertion of foreign DNA into the cloning site located in the lacZ' gene results in inactivation of beta-galactosidase.

Approximately 80% of the JM105 transformants obtained from this procedure contained recombinant plasmids as indicated by the inactivation of the lacZ' gene (lacZ'.sup.-), i.e., the transformants did not produce beta-galactosidase.

To screen for lysostaphin expression in the transformants, *S. aureus* RN492, described by Novick et al., Plasmid, Vol. 2, pp. 109-129 (1979) and obtained therefrom, was used as the indicator strain. This bacterial strain is a constitutive beta-lactamase producer and is relatively resistant to ampicillin. *E. coli* JM105 transformants grown on L agar containing 50 μ g/ml ampicillin were exposed to chloroform vapor for 30 minutes to lyse them and overlaid with a 0.1% (v/v) suspension of a stationary phase culture of *S. aureus* RN492 in GL agar. Production of lysostaphin by *E. coli* JM105 transformants, indicating the successful insertion of the lysostaphin gene into pUC8, was determined by observation of lysis of the indicator cells superimposed on a JM105 transformant colony. Approximately nine out of 1000 clones harboring recombinant plasmids (amp.sup.+, lacZ'.sup.-) contained the lysostaphin gene, a number which suggested that multiple copies of the lysostaphin gene were present per chromosomal equivalent of *S. simulans* DNA (about 2000 kbp).

Lysostaphin-producing transformants contained recombinant plasmids having inserts of 6.0, 6.5, or 8.0 kbp. Restriction analysis showed that these inserts were present in either orientation in the cloning vector and contained a 4.3 kbp DNA fragment in common. The DNA sequence coding for lysostaphin was further localized to a 1.5 kbp Hpa II-Hind III DNA fragment obtained from the 4.3 kbp DNA fragment. This 1.5 kbp DNA fragment was recloned into the Acc I-Hind III sites of pUC8 to create the recombinant plasmid pRG5, which is a preferred embodiment of this invention.

EXAMPLE 2

SerAlaGlyTyrGlyLysAlaGlyGlyThrValThrProThrProAsnThrGlyTrpLys
 1141: aacaaacaaatatggcacactatataaatcagagtcagcttagcttcacacctaatacaga
 ThrAsnLysTyrGlyThrLeuTyrLysSerGluSerAlaSerOheThrProAsnThrAsp
 1201: tataataacaagaacgactgggtccatttagaagcatgccgcagtcaggagtccttaaaagc
 IleIleThrArgThrThrGlyProPheArgSerMetProGlnSerGlyValLeuLysAla
 1261: aggtcaaacaattcattatgatgaagtgatgaaacaagacgggtcatgtttgggtaggtta
 GlyGlnThrIleHisTyrAspGluValMetLysGlnAspGlyHisValTrpValGlyTyr
 1321: tacaggtaacagtggccaacgtatttacttgccgtgaagaacatggaataaatctactaa
 ThrGlyAsnSerGlyGlnArgIleTyrLeuProValArgThrTrpAsnLysSerThrAsn
 1381: tacttttagtgtcttttgggggaactataaaaagtgagcgcgctttttataaacttatatgat
 ThrLeuGlyValLeuTrpGlyThrIleLys
 1441: aattagagcaaataaaaaattttttctcattcctaaagttgaagc

The amino terminal 36 amino acid sequence of preprolystaphin is the signal peptide, i.e. the largely hydrophobic region found in precursors of secreted proteins.

Signal peptides are the amino terminal sequences of secreted proteins as translated which are involved in directing the growing polypeptide chain through the membrane after synthesis on membrane-bound ribosomes. In eukaryotes, the signal peptide is cleaved off by a specific protease found in the luminal side of the rough endoplasmic reticulum (RER), even before polypeptide synthesis is completed.

In bacteria, which do not have an RER, secreted proteins are synthesized on ribosomes which may be bound to the internal face of the plasma membrane. The signal peptide of nascent bacterial polypeptide chains appears to be involved in transporting the protein through the plasma membrane. Bacterial signal peptides, in general, are removed during or shortly after transport through the plasma membrane, since secreted proteins that accumulate in culture medium no longer contain this sequence.

Several features of preprolystaphin can be seen with reference to Formula II which depicts the amino terminal 152 amino acids of preprolystaphin (A) and the nucleotide sequence of the 1.5 kbp DNA fragment from base pairs 389-661 (B).

The signal peptide or signal sequence of secreted or membrane bound proteins is characterized by a high content of hydrophobic amino acids as is apparent in the signal peptide of preprolystaphin shown in Formula IIA. Although the hydrophobicity of any signal sequence is a constant finding, the actual amino acids contained therein, the particular sequence of the amino acids and the length of the peptide can vary widely. For example, usually long signal sequences (31-44 amino acids) occur in *Bacillus* species. Watson, *Nucleic Acids Res.*, Vol. 12, pp. 5145-5164, 1984.

The cleavage site of the preprolystaphin signal sequence is the Ala-Ser bond at amino acid residues 36-37 (Formula IIA). Removal from the 1.5 kbp DNA fragment of the DNA coding for the signal sequence of preprolystaphin would most likely result in a non-secreted protein. A DNA sequence coding for a functionally equivalent signal peptide which replaces the DNA encoding the lysozyme signal peptide would also most likely encode a secretable "preprolystaphin," albeit one with a different, but functional signal peptide.

Following cleavage of the signal sequence which is required for secretion, the protein that accumulates and is subsequently processed is prolystaphin. Also shown in Formula IIA is the sequence from Ala-49 through ARG-139 of prolystaphin which contains seven tandem repeats of a 13 amino acid sequence. This portion of the protein, which contains a large amount of glutamic acid and has a net negative charge, is cleaved off during the processing of prolystaphin to mature lysozyme. The DNA sequence, i.e. bp 389 to 661, which codes for the amino acid repeats

is composed of seven repeats of an homologous 39 bp sequence. (Formula IIB). The seven tandem amino acid repeats (IIA) and corresponding nucleotide sequence repeats (IIB) are numbered 1-7 in Formula II.
##STR1##

A wide variety of proteins are synthesized in precursor forms with subsequent processing to mature active proteins. For example, insulin, which in its active form is a two chain polypeptide, is synthesized as a single polypeptide chain (proinsulin) that is subsequently converted to mature insulin following proteolytic removal of an internal polypeptide. Also, the alkaline and neutral proteases of *B. subtilis* and *B. amyloliquefaciens* are synthesized as preproenzymes. It has not been previously known though that lysostaphin was synthesized as a preproenzyme. As shown in Table I, conversion of the alkaline and neutral protease proenzymes to mature enzymes involves a similar cleavage site as does the conversion of prolysostaphin to lysostaphin.

TABLE I

Comparison of Preprolysostaphin

With *Bacillus* Preproproteases

	Number of		
	Residues		
	Prepro		
	Se-	Ma-	
Enzyme	quence	ture	Final Cleavage Site
<hr/>			
Subtilisin			
B. subtilis			
	106	275	HisGluTyr .downarw. AlaGlnSerVal
B.	107	275	HisAlaTyr .downarw. AlaGlnSerVal
amyloliquefaciens			
Neutral Protease			
B. subtilis			
	221	300	ValGluHis .downarw. AlaAlaAlaThr
B.	221	300	ValGluHis .downarw. Ala AlaThrThr
amyloliquifaciens			
Lysostaphin			
	143	246	AlaLeuArg .downarw. AlaAlaThrHis

The amino terminal sequence of purified mature lysostaphin from *S. simulans* determined by Edman degradation, Ala-Ala-Thr-His-Glu, corresponds to amino acids 144-148 of the preprolysostaphin sequence

EXAMPLE 3

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weight of 64,000 daltons was also seen in the *E. coli* cellular fraction (FIG. 1, lane 7). A cross-reactive protein with electrophoretic mobility identical to this larger protein was also seen in supernatants of *S. simulans*, harvested primarily after growth to late log phase. The larger cross-reacting protein is, most likely, prolysostaphin, since in both *S. simulans* and *E. coli* JM105/pRG5, it precedes lysostaphin in appearance and disappears as mature lysostaphin accumulates (FIG. 1, lanes 1-4 and 8). The apparent overestimation of the molecular weight of prolysostaphin most likely results from decreased binding of SDS due to the high content of glutamic acid residues in the tandem repeats of the prolysostaphin sequence.

Lysostaphin, therefore, is synthesized as a preproenzyme. The signal sequence of the preprolysostaphin is cleaved upon vectorial transport of the polypeptide chain through the membrane. The resulting prolysostaphin is subsequently processed extracellularly to mature lysostaphin. Conversion to the mature active enzyme is accomplished by cleavage of the Arg.sub.143 -Ala.sub.144 peptide bond. The amino terminal portion of prolysostaphin apparently is removed in stages, i.e., the entire proenzyme sequence is not all removed at the same time. This processing reaction occurs in the supernatant of stationary phase cultures of *S. simulans*. Since mature enzyme is also produced by cultures of *E. coli* JM105/pRG5 transformants, the proenzyme processing is either autocatalytic or involves similar processing activities which are present in both *E. coli* JM105 and *S. simulans*. *E. coli* JM105/pRG5 transformants, however, have the advantage that processing appears to occur intracellularly rather than extracellularly as in *S. simulans*.

EXAMPLE 4

Construction of Plasmids Which in Transformant *Bacillus* spp. Express Lysostaphin

Bacillus expression systems for production of cloned gene products have significant advantages over similar systems using *E. coli* as the host organism. *Bacillus* species normally secrete proteins readily into the surrounding medium. Because of this advantage, recombinant plasmids containing the 1.5 kbp DNA sequence coding for lysostaphin were constructed from plasmids that replicate in various *Bacillus* species.

Plasmid pRG5 was used as the source of the DNA encoding lysostaphin. Plasmid pRG5 DNA, digested with Hind III and Eco RI according to the conditions specified by the manufacturer, was fractionated by preparative electrophoresis in 1% agarose. The 1.5 kbp DNA fragment which codes for lysostaphin was located by ethidium bromide staining and transferred by electrophoresis to a DEAE-nitrocellulose filter strip. The strip was washed with NET buffer (0.15M NaCl, 0.1 mM EDTA, 0.02 MTris, pH 8.0) and the bound DNA was eluted by incubation of the strip in NET buffer containing 1M NaCl for 1 hour at 65.degree. C. Ethidium bromide was removed from the DNA by twice extracting the solution with an equal volume of n-butanol. DNA was precipitated by addition of two volumes of cold 95% ethanol to the aqueous phase, collected by centrifugation, washed with 80% ethanol and dissolved in TE buffer (Example 1).

a. Plasmid pJP1

Recombinant plasmid, pJP1, a preferred embodiment of this invention, was constructed by inserting the 1.5 kbp DNA fragment coding for lysostaphin into the *Bacillus* plasmid pBC16, which carries the gene for tetracycline resistance. Plasmid pBC16, obtained from the collection of Richard P. Novick, Public Health Research Institute, New York, N.Y., was isolated from soil bacilli and is highly homologous to and incompatible with pUB110, a *S. aureus* plasmid specifying kanamycin resistance. pBC16 DNA was isolated from *B. subtilis* by the alkaline-SDS procedure

described by Birnboim, Meth. Enzymol, Vol. 100, pp. 243-255 (1983). Cells from a 250 ml overnight culture grown in VY medium (25 g Difco veal infusion; 5 g Difco yeast extract/1 H.sub.2 O) were harvested by centrifugation, washed in TE buffer and resuspended in 5 ml of TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0). Lysozyme (1 mg/ml) was added and the suspension was incubated at room temperature for 20 minutes. After addition of 10 ml of 0.2% NaOH-1% SDS, the mixture was incubated at 0.degree. C. for 45 minutes. Ten ml of 3M potassium acetate-1.8M formic acid were then added and the mixture was further incubated at 0.degree. C. for 30 minutes. The lysate thus obtained was centrifuged at 15,000.times.g for 20 minutes. Two volumes of 95% ethanol were added to the supernatant and the resulting precipitate, obtained after 15 minutes at room temperature, was collected by centrifugation at 10,000.times.g for 10 minutes, washed with 80% ethanol and dissolved in 0.5 ml TE buffer. Approximately 200 .mu.g of closed circular pBC16 DNA was obtained with this procedure.

The plasmid DNA was linearized by Eco RI cleavage. A mixture of linearized pBC16 DNA (approximately 1 .mu.g) and the 1.5 kbp DNA fragment coding for lysostaphin (about 1 .mu.g) was blunt ended using DNA polymerase (Klenow fragment). T4 DNA ligase was then used to ligate the plasmid and fragment DNA together, thereby reforming closed circular plasmid DNA molecules.

Competent *B. subtilis* strain BD 170 cells, obtained from David Dubnau of Public Health Research Institute, New York, N.Y., were transformed with the ligated DNA (approximately 1 .mu.g DNA per 0.1 ml of cells) according to the method of Contente et al., Mol. Gen. Genet., Vol. 167, pp. 251-258 (1979). The cells were then incubated at 37.degree. C. for 90 minutes to allow expression of the plasmid's tetracycline resistance gene and plated on TBAB agar containing a selective amount of tetracycline (5 .mu.g/ml) and suspended heat-killed *S. aureus* cells to indicate lysostaphin production.

Heat-killed *S. aureus* cells for measuring lysostaphin activity were prepared by autoclaving (40 min.) a stationary phase culture grown in 500 ml CYGP broth. The dead cells were centrifuged and resuspended in 10 ml sterile water. Two ml of this dead cell preparation was added to 500 ml TBAB agar for preparation of the indicator plates.

Approximately 1% of the *B. subtilis* cells transformed with the ligated DNA produced lysostaphin as indicated by the lysis of staphylococcal cells surrounding the *B. subtilis* colonies. pJPl was obtained by restreaking one of the *B. subtilis* transformants on lysostaphin indicator plates several times until a completely stable clone was obtained.

Lysostaphin activity was present primarily in the supernatant fraction of *B. subtilis* BD 170/pJPl transformants grown to stationary phase in liquid medium. Immunoblot analysis showed that a lysostaphin precursor form was secreted which was subsequently converted to mature lysostaphin. In addition, immunoblotting also showed the presence of lysostaphin degradation products in culture supernatants which were minimized when phenylmethylsulfonyl fluoride, a serine-protease inhibitor, was added to the culture. It is thus likely that a serine-protease is secreted into the medium or present on the surface of *B. subtilis* BD170 cells.

b. pDF8 and pRP1

Two other recombinant plasmids, pDF8 and pRP1, containing the lysostaphin gene were constructed for transformation of *Bacillus* species essentially as provided in Example 4a for the construction of pJPl.

pDF8 was obtained by inserting the 1.5 kbp DNA fragment coding for

lysostaphin into the Eco RI restriction site of plasmid pBD64, a kanamycin, chloramphenicol resistance plasmid obtained from the collection of David Dubnau, Public Health Research Institute, New York, N.Y. pDF8 was obtained, as above, following transformation of *B. subtilis* BD 170 with recircularized plasmids and planting such transformants on lysostaphin indicator plates containing 5 $\mu\text{g/ml}$ chloramphenicol. pDF8 was selected following repeated restreaking of positive clones.

Likewise, pRP1 was constructed by inserting the 1.5 kbp DNA fragment which code for lysostaphin into the HpaI site of pSPV1, a chloramphenicol resistance plasmid obtained from Steven Projan, Public Health Research Institute, New York, N.Y. All subsequent steps for isolation of pRP1 were identical to those for the construction of pJP1 and pDF8.

EXAMPLE 5

Lysostaphin Expression in *B. sphaericus* 00/pJP1 Transformants

A number of *Bacillus* strains were transformed with pJP1 to obtain a suitable host for large scale production of lysostaphin. Transformants which produced large lysis halos on lysostaphin indicator plates were isolated and further characterized. Of the *Bacillus* strains screened, *B. sphaericus* strain 00, an organism isolated and maintained in the culture collection of the Public Health Research Institute, New York, N.Y., provides maximal production of lysostaphin.

B. sphaericus 00/pJP1 transformants were obtained by treating protoplasts obtained by lysozyme treatment of intact cells with pJP1 DNA in the presence of polyethylene glycol according to the method of Chang et al., *Molec. Gen. Genet.*, Vol. 168, pp. 11-115 (1979). Following treatment, the cells were grown on DM3 regeneration plates in the presence of tetracycline (5 $\mu\text{g/ml}$) prior to assay on lysostaphin indicator plates.

B. sphaericus 00/pJP1 transformants grown on VY or CYGP medium produced and secreted approximately 150 mg mature active lysostaphin per liter of culture medium. This amount is about five times the amount produced by *S. simulans* under the best fermentation techniques currently available. Active lysostaphin accumulates in the growth medium with little degradation, even after prolonged incubation of cultures, and accounts for approximately 80% of total extracellular protein. Lysostaphin isolated from *B. sphaericus* 00/pJP1 transformants is immunologically, electrophoretically and catalytically indistinguishable from that obtained from *S. simulans* cultures. Lysostaphin is isolated from the growth medium in accordance with known fractional precipitation (salting out) procedures. Alternatively, a particularly effective purification is achieved by combining a precipitation and a chromatographic separation of the fermentation broth from cultures of lysostaphin-producing *B. sphaericus* 00/pJP1 transformants.

Cells are removed from the fermentation broth, for example by centrifugation or ultrafiltration, and solid ammonium sulfate is added to the supernatant to 40-60%, preferably 50%, of saturation. After 1 hour at 4 degree C., the lysostaphin-containing precipitate is recovered by centrifugation. Recovery at this step is greater than 80%.

The precipitate is redissolved in a minimum volume of 10 mM sodium phosphate buffer (pH 7.00, 50 mM NaCl) and dialyzed against 100 volumes of the same buffer. After removal of any particulate material, the dialyzed solution is chromatographed on a cation exchange column (preferably Pharmacia FPLC Mono S) and eluted using a buffered gradient of increasing salt concentration from 0.05 to 0.25M NaCl. Recovery of lysostaphin for the single chromatographic step was more than 90%.

Lysostaphin activity is associated with two major peaks. The later eluting peak of lysostaphin is comprised of non-covalent aggregates of the protein. These aggregates dissociate on dilution in buffer and under conditions of sodium dodecylsulfate polyacrylamide gel electrophoresis.

The lysostaphin that is purified from the culture medium of *B. sphaericus* 00/pJP1 transformants is substantially free of non-lysostaphin contaminants. Of special significance is that the *B. sphaericus* 00/pJP1 lysostaphin is substantially free of immunogenic staphylococcal contaminants.

CLAIMS:

I claim:

1. Recombinant plasmids containing a DNA sequence which codes for lysostaphin and which in transformant microbial hosts will express a gene encoding lysostaphin from *S. simulans* (NRRLB-2628).
2. Recombinant plasmids according to claim 1, in which the recombinant plasmid is selected from the group consisting of pRG5, pJP1, pDF8, and pRP1.
3. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pRG5.
4. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pJP1.
5. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pDF8.
6. A recombinant plasmid according to claim 1 in which the recombinant plasmid is pRP1.
7. Transformant microorganism which produces lysostaphin, the microorganism being transformed by a recombinant plasmid containing a DNA sequence which codes for lysostaphin from *S. simulans* (NRRL B-2628).
8. Transformant microorganisms according to claim 7, in which the microorganisms are selected from the group consisting of *E. coli*, Yeast, *Streptomyces* spp., and *Bacillus* ssp.
9. Transformant microorganisms according to claim 7, in which the microorganisms are selected from the group consisting of *E. coli*, *B. subtilis* and *B. sphaericus*.
10. Transformant microorganism according to claim 7, in which the microorganism is *E. Coli* K-12 strain JMI05.
11. Transformant microorganism according to claim 10, in which the recombinant plasmid is pRG5.
12. Transformant microorganism according to claim 7, in which the microorganism is *B. sphaericus* strain 00.
13. Transformant microorganism according to claim 12, in which the recombinant plasmid is pJP1.
14. Transformant microorganism according to claim 7, in which the microorganism is *B. subtilis* BD170.
15. Transformant microorganism according to claim 14, in which the recombinant plasmid is pJP1.

16. Transformant microorganism according to claim 14, in which the recombinant plasmid is pDF8.

17. Transformant microorganism according to claim 14, in which the recombinant plasmid is pRP1.

18. A method of expressing lysostaphin comprising transforming a microorganism by a recombinant plasmid which contains a DNA sequence which codes for lysostaphin from *S. simulans* (NRRL B2628) and culturing the transformed microorganism under conditions suitable for growth and expression of lysostaphin.

19. A DNA fragment selected from the group consisting of:

(a) a 1.5 kilobase DNA fragment coding for lysostaphin having the following amino acid sequence which comprises the following nucleotide sequence

1: ccggaactcttgaatgttttagttttgaaaattccaaaaaaacctacttttcttaattatt
61: gattcatattattttaacacaatcagtttagaatttcaaaaatcttaaagtcaatttttga
121: gtgtgtttgtatattttcatcaaaatcaatcaatattttttacttttcttcatcgttaaaa
181: aatgtaataatttataaaaaatgctatttctcataaatgtaataataaattaggaggtatt
241: aaggttgaagaaaaacaaaaaacaattattatacagagaccttttagctattggactgagtac
f-MetLysLysThrLysAsnAsnTyrTyrThrArgProLeuAlaIleGlyLeuSerThr
301: atttgccttagcatctattgtttatggagggtattcaaaatgaaacacatgcttctgaaaa
PheAlaLeuAlaSerIleValTyrGlyGlyIleGlnAsnGluThrHisAlaSerGluLys
361: aagtaatatggatgtttcaaaaaagtagctgaagtagagacttcaaaagccccagtaga
SerAsnMetAspValSerLysLysValAlaGluValGluThrSerLysAlaProValGlu
421: aaatacagctgaagtagagacttcaaaagctccagtagaaaaatacagctgaagtagagac
AsnThrAlaGluValGluThrSerLysAlaProValGluAsnThrAlaGluValGluThr
481: ttcaaaagctccagtagaaaaatacagctgaagtagagacttcaaaagctccagtagaaaa
SerLysAlaProValGluAsnThrAlaGluValGluThrSerLysAlaProValGluAsn
541: tacagctgaagtagagacttcaaaagctccggtagaaaaatacagctgaagtagagacttc
ThrAlaGluValGluThrSerLysAlaProValGluAsnThrAlaGluValGluThrSer
601: aaaagccccagtagaaaaatacagctgaagtagagacttcaaaagccctggttcaaaatag
LysAlaProValGluAsnThrAlaGluValGluThrSerLysAlaLeuValGlnAsnArg
661: aacagctttaagagctgcaacacatgaacattcagcacaatgggtgaataattacaaaaa
ThrAlaLeuArgAlaAlaThrHisGluHisSerAlaGlnTrpLeuAsnAsnTyrLysLys
721: aggatatgggtacgggtccttatccattaggtataaatggcggtatgcactacggagttga
GlyTyrGlyTyrGlyProTyrProLeuGlyIleAsnGlyGlyMetHisTyrGlyValAsp
781: tttttttatgaatattggaacaccagtaaaagctatttcaagcggaaaaatagttgaagc
PhePheMetAsnIleGlyThrProValLysAlaIleSerSerGlyLysIleValGluAla
841: tgggttgagtaattacggaggaggtaatcaaataaggtcttattgaaaatgatggagtga
GlyTrpSerAsnTyrGlyGlyGlyAsnGlnIleGlyLeuIleGluAsnAspGlyValHis
901: tagacaatggtatattgcatctaaagtaataatgttaaagtaggagattatgtcaaagc
ArgGlnTrpTyrMetHisLeuSerLysTyrAsnValLysValGlyAspTyrValLysAla
961: tgggtcaaataatcggttggtctggaagcactggttattctacagcaccacatttacactt
GlyGlnIleIleGlyTrpSerGlySerThrGlyTyrSerThrAlaProHisLeuHisPhe
1021: ccaagaatgggttaattcattttcaaaattcaactgcccaagatccaatgcctttcttaaa
GlnArgMetValAsnSerPheSerAsnSerThrAlaGlnAspProMetProPheLeuLys
1081: gagcgcaggatatggaaaagcaggtggtacagtaactccaacgccgaatacaggttgga
SerAlaGlyTyrGlyLysAlaGlyGlyThrValThrProThrProAsnThrGlyTrpLys
1141: aacaaacaaatattggcacactatataaatcagagtcagctagcttcacacctaatacaga
ThrAsnLysTyrGlyThrLeuTyrLysSerGluSerAlaSerPheThrProAsnThrAsp
1201: tataataacaagaacgactggtccatttagaagcatgccgcagtcaggagctttaaagc
IleIleThrArgThrThrGlyProPheArgSerMetProGlnSerSlyValLeuLysAla
1261: aggtcaaacaattcattatgatgaagtgatgaaacaagacgggtcatgtttgggtaggtta
GlyGlnThrIleHisTyrAspGluValMetLysGlnAspGlyHisValTrpValGlyTyr
1321: tacaggtaacagtgcccaacgtatttacttgctgtaagaacatggaataaatctactaa
ThrGlyAsnSerGlyGlnArgIleTyrLeuProValArgThrTrpAsnLysSerThrAsn
1381: tacttttaggtgttcttttggggaactataaagtgagcgcgctttttataaacttatatgat
ThrLeuGlyValLeuTrpGlyThrIleLys
1441: aattagagcaaataaaaaattttttctcattcctaaagttgaagc,

(b) A DNA fragment coding for mature lysostaphin comprising nucleotides 674-1411 in (a),


(c) a DNA fragment coding for preprolysostaphin comprising nucleotides 245-1411 in (a),

- (d) a DNA fragment coding for prolystostaphin comprising nucleotides 356-1411 in (a), and
(e) a DNA fragment coding for the lysostaphin signal peptide comprising nucleotides 245-355 in (a).

20. The DNA fragment of claim 19 coding for mature lysostaphin.
21. The DNA fragment of claim 19 coding for prepolysostaphin.
22. The DNA fragment of claim 19 coding for prolystostaphin.
23. The DNA fragment of claim 19 coding for the lysostaphin signal peptide.

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